VOL. 53 NO. 12, DEC. 2000 THE JOURNAL OF ANTIBIOTICS pp. 1341 — 1345

FR191512, a Novel Anti-influenza Agent Isolated from a Fungus Strain No.17415

II. Biological Properties

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(Recieved for publication July 6, 2000)

FR191512, a novel polyphenolic compound, inhibited the infectivity of influenza A virus in Madin-Darby canine kidney (MDCK) cells *in vitro*. Furthermore, FR191512 showed good *in vivo* anti-influenza activity in a mouse model of intranasal infection with influenza A virus. The cytotoxic activity of FR191512 against MDCK cells was very weak.

The sialidase (neuraminidase) of influenza virus is known to promote the release of progeny virus from infected cells, and the selective sialidase inhibitor zanamivir (GG167) has been shown to inhibit the growth of influenza virus *in vitro* and *in vivo*[1~4].

To discover a novel compound that inhibits infectivity of influenza virus, we attempted to screen new viral sialidase inhibitors from microbial secondary metabolites. As a result, we isolated a novel anti-influenza viral agent FR191512 of fungus origin. Its taxonomy, fermentation, isolation, physico-chemical properties and structure elucidation have been already reported in the preceding paper[5]. In this report we describe the biological properties of FR191512 *in vitro* and *in vivo*.

Materials and Methods

Compounds and Reagents

Ribavirin was purchased from Viratek, a subsidiary of ICN Pharmaceuticals Inc., through the United Kingdom distributor, Britannia Pharmaceuticals Ltd., Redhill, Surrey. 4-Guanidino-Neu5Ac2en (zanamivir, GG167) was synthesized from Neu5Ac2en in the Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., Tsukuba, Japan. Newcastle disease virus sialidase and Yeast α-glucosidase were purchased from Sigma Chemical Co. (St Louis, MO, USA). *Streptococcus* 6646K sialidase was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan).

In Vitro Anti-influenza Activity (Plaque Assay)

MDCK cells and influenza A/PR/8/34 (H1N1) virus for plaque assay were supplied from Dr. NEROME, the National Institute of Infectious Diseases, Tokyo, Japan. Plaque assay was performed by a modification of the method described by HAYDEN *et al.*[7]. MDCK cells were inoculated with influenza virus diluted in EAGLE's modification of minimal essential medium (pH 7.2~7.4) containing 1 μg of TPCK-treated trypsin and test compound to give 100 to 200 plaques per well (6-well plate). Cells were left for 1 hour at 37°C for virus to adsorb and overlaid with defined cell growth medium containing 1% Noble agar (Sigma Chemical Company), 1 μg of TPCK-treated trypsin per ml, 0.001% DEAE dextran, and test compound. After cells were incubated for 48 hours at 37°C (humidified 5% CO_2), plaques were visualized by staining viable cells with neutral red. The percent inhibition of plaque formation relative to controls (in the absence of any inhibitor) was calculated for each inhibitor concentration.

In Vivo Efficacy

FR191512 was evaluated in a mouse model of respiratory tract infection. Mouse-adapted influenza A/PR/8/34 virus was used in this study. Briefly, BALB/c

Table 1. *In vitro* anti-influenza activity of FR191512, zanamivir and ribavirin by plaque formation inhibition assay.

Compound	IC_{50} (μM)	IC_{80} (μM)
FR191512	0.28	0.92
Zanamivir	0.10	0.87
Ribavirin	6.40	15.8

mice (female, four weeks old) were anesthetized by the intravenously administration at 1.0 mg/kg with pentobarbiturate and were inoculated intranasally with 20 μl of virus suspension (5.1~10^2 PFU/mouse). And then mice were anesthetized by the inhalation of ether and were dosed intranasally twice daily with test compounds from the day before infection to 3 days after infection. The efficacies of anti-influenza agents were assessed at the 50% effective dose (ED_{50}) calculated by probit analysis on the survival rate at 7 days after infection.

Sialidase Assay

Influenza virus sialidase activity was measured by a modification of the method described by POTIER *et al.*[6]. Equal volumes of test compound and virus were mixed and preincubated at 37°C for 20 minutes in 96-well microtiter plate.

The reaction was initiated by the addition of buffer (32.5 mM MES, pH 6.5) containing 100 μM 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (Sigma Chemical Company). The reaction mixture was incubated at 37°C for 1 hour, and the reaction was terminated by the addition of 100 μl of 0.02 N NaOH in 80% ethanol. 4-Methylumbelliferone was immediately measured by spectrofluorometer (Fluoroskan, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). The excitation wavelength and the emission wavelength was set at 365 nm and 450 nm, respectively. The percent inhibition relative to positive (no test compound) controls was calculated.

Cytotoxicity Test

The cytotoxic activity of FR191512 against MDCK cells was compared with that of zanamivir and ribavirin. Concentration of the test compound required for 50% inhibition of cell growth (CC_{50}) was determined. The cytotoxicity was colorimetrically determined at 550 nm (and 660 nm as a reference) according to MTT method[8,9].

Results

In Vitro Anti-influenza Activity

The *in vitro* anti-influenza virus activity of FR191512 was compared with that of ribavirin and zanamivir using plaque formation inhibition assay (Table 1). In this study, the antiviral effect of inhibition has been quantified by reduction in plaque numbers.

The results of inhibition of plaque formation in MDCK cells of influenza A/PR/8/34 virus are shown in Table 1. The IC_{50} value of FR191512 was 0.28 μM. FR191512 appeared to be about 20-fold more active than ribavirin (IC_{50} of 6.40 μM) but was 3-fold less effective than zanamivir (IC_{50} of 0.10 μM). However, the IC_{80} of FR191512 was 0.92 μM and the value was almost equal to that of zanamivir (IC_{80} of 0.87 μM).

In Vivo Activity

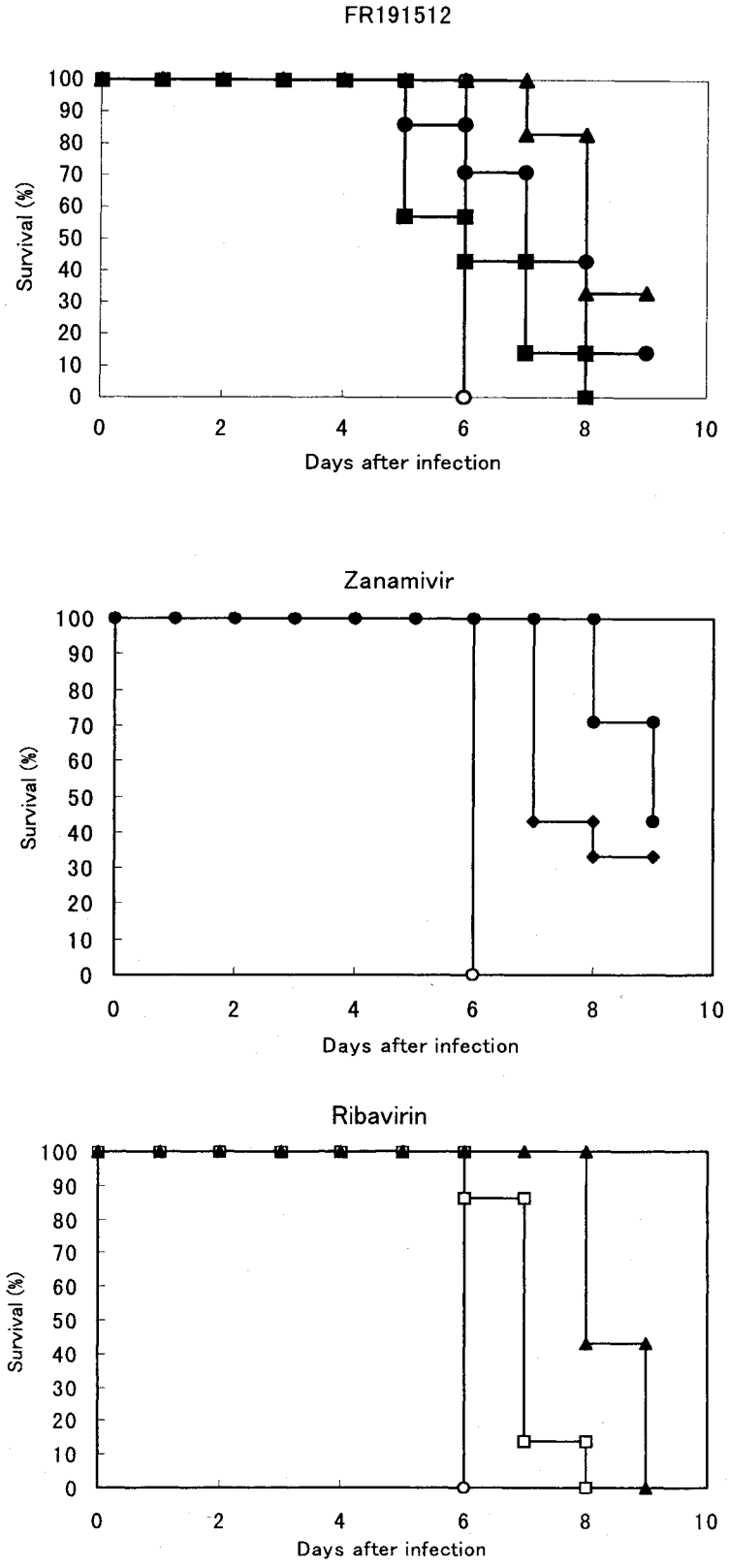
The protective efficacy of FR191512, ribavirin and zanamivir in a mouse model of respiratory tract infection with influenza A/PR/8/34 virus was examined (Fig. 1). These compounds prolonged the survival of infected mice. The ED_{50}s of FR191512, ribavirin and zanamivir at day 7 after challenge were 3.20, 16.2 and 1.16 mg/kg, respectively. The result demonstrates that *in vivo* efficacy of test compounds was nearly identical with *in vitro* anti-influenza virus activity. Thus, FR191512 is slightly less potent than zanamivir but more effective than ribavirin.

Inhibitory activities against various sialidase and other glycosidases.

Inhibitory activities of FR191512 and zanamivir against influenza virus sialidase and other glycosidases were examined. The IC_{50}s of them were listed in Table 3. Zanamivir selectively inhibited influenza virus sialidase. On the other hand, FR191512 showed inhibition against newcastle disease virus sialidase, *Streptococcus* 6646K

Fig. 1. Protective effect of FR191512, zanamivir and ribavirin in intranasal infection of influenza A/PR/8/34 virus in mice.

The test compound was administered intranasally twice daily for 3 days.



Symbols: ▲, 32 mg/kg; □, 10 mg/kg; ●, 3.2 mg/kg; ◆, 1.0 mg/kg; ■, 0.32 mg/kg; ○, Vehicle.

Table 2. *In vivo* anti-influenza activity against of FR191512, zanamivir and ribavirin in a murine model.

Compound	ED_{50} (mg/kg) [a]
FR191512	3.20
Zanamivir	1.16
Ribavirin	16.2

Tested compound was intranasally administered to BALB/c mice (n=7) infected with influenza A /PR/8/34 virus.
[a] The ED_{50} was assessed at 7 days after infection.

Table 4. Cytotoxicity against MDCK cells *in vitro*.

Compound	CC_{50} (μM)	SI[a]
FR191512	167	596
Zanamivir	>300	>3125
Ribavirin	64.0	10.0

[a] SI (Selectivity index) was calculated by the formulation of CC_{50}/(IC_{50} in plaque assay)

Table 3. Inhibitory activities against various sialidases and other glycosidases.

Glycosidase	IC_{50} (μM)	
	FR191512	Zanamivir
Sialidase (Influenza virus A/PR/8)	33.3	0.093
Sialidase (Newcastle disease virus)	2.08	>300
Sialidase (*Streptococcus* 6646K)	2.08	>300
α-Glucosidase (Yeast)	2.08	>300

sialidase and yeast α-glucosidase.

Cytotoxic Activity

As shown in Table 4, the cytotoxic activity of FR191512 against MDCK cells was weaker than ribavirin. The selective index value (CC_{50}/IC_{50} in plaque assay) of FR191512 was 60-fold higher than ribavirin.

Discussion

The *in vitro* and *in vivo* anti-influenza virus activities of FR191512 were evaluated and compared with those of other antiviral agents. FR191512 showed potent anti-influenza virus activity *in vitro* using plaque formation inhibition assay. The activity of FR191512 was more potent than ribavirin but was less effective than zanamivir in assessment of the IC_{50} value. However, the IC_{80} value of FR191512 was almost equal to that of zanamivir. The results of plaque assay suggest that FR191512 has a strong anti-influenza efficacy. Furthermore, FR191512 showed a potent protective efficacy in a murine model of respiratory tract infection with influenza virus. The *in vivo* efficacy of FR191512 nearly reflected *in vitro* anti-influenza virus activity.

The structure of FR191512 was determined as a novel polyphenolic compound. There are some reports concerning anti-influenza viral activities of polyphenol compounds from plant leaf extracts[10]. In these reports, tea polyphenols have inhibited the viral sialidase weakly but have strongly inhibited infectivity of influenza virus to MDCK cells by blocking its adsorption and entry into the cells. These knowledge may correlate closely with the results that FR191512 has weak viral sialidase inhibition activity but its anti-influenza viral efficacies *in vitro* and *in vivo* are strong. In the present study, we have not ever examined the mechanism of action for FR191512 until now. However, FR191512 may show the same mechanism of action as these polyphenols in our estimation.

Acknowledgement

The authors thanks Prof. Y. SUZUKI, Department of Biochemistry, School of Pharmaceutical Science, University of Shizuoka, for critical reviewing this manuscript.

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